



TITLE:

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CITATION:

Sakuradani, Eiji ...[et al]. Subterminal oxidation of n-alkanes in achlorophyllous alga *Prototheca* sp.. *Journal of bioscience and bioengineering* 2013, 116(4): 472-474

ISSUE DATE:

2013-10

URL:

<http://hdl.handle.net/2433/178747>

RIGHT:

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Note

Subterminal oxidation of *n*-alkanes in achlorophyllous alga *Prototheca* sp.

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1 **Abstract**

2 Some *Prototheca* sp. are known to be involved in *n*-hexadecane degradation. Two
3 derivatives derived from *n*-hexadecane in such *Prototheca* sp. were identified as
4 5-hexadecanone and 5-hexadecanol. *n*-Hexadecane was assumed to be converted to
5 5-hexadecanol and then to 5-hexadecanone through a unique subterminal oxidation
6 pathway in such *Prototheca* sp.

7

8 **Keywords**

9 Prototheca; *n*-alkane; subterminal oxidation; secondary alcohol; ketone

The genus *Prototheca* comprises achlorophyllous heterotrophic microorganisms closely related to a photosynthetic green alga, *Auxenochlorella protothecoides*, on the phylogenetic tree of 18S rDNA sequences (1). In the 1970s, *Prototheca hydrocarborea* was firstly found to utilize hydrocarbons in crude oil for growth by Kocková-Kratochvílová and Havelková (2). Walker et al. (3-5) reported that a *P. zopfii* strain isolated from Chesapeake Bay grew on crude oil and mixed hydrocarbons, being able to utilize a large amount of crude oil as carbon sources in comparison with in the case of the hydrocarbon heterotrophy between *P. zopfii* and some crude oil-degrading bacteria. Koenig and Ward (6) established optimum conditions as to pH, temperature, and salinity for acetate-supported growth of a new isolate, *P. zopfii* Krüger strain UMK-13. Ueno et al. (7) isolated a thermotolerant strain, *P. zopfii* RND16, which assimilated *n*-alkanes at 38°C and grew well on a mixture of *n*-alkanes and polycyclic aromatic hydrocarbons at 35°C, and established a means of repeated batch cultivation of *P. zopfii* RND16 immobilized in polyurethane foam for bioremediation.

n-Alkanes are assimilated by a wide variety of microorganisms such as bacteria, yeasts, filamentous fungi, and algae. Two major pathways for *n*-alkane degradation are known (8): the terminal oxidation pathway, which is more general among microorganisms, and the subterminal oxidation pathway, which, in contrast, has been found in limited examples reported. Subterminal oxidation involves the hydroxylation at a middle carbon in an *n*-alkane, a secondary alcohol being generated that is then oxidized by an alcohol dehydrogenase to the corresponding ketone. The ketone is

converted to an ester by a Baeyer-Villiger monooxygenase, that is then cleaved into a primary alcohol and a fatty acid by an esterase. The primary alcohol is oxidized to the corresponding fatty acid and then enters the β -oxidation pathway.

Although *P. hydrocarbonea* and *P. zopfii* are well-known to assimilate hydrocarbons, the metabolic pathway for hydrocarbons has not been elucidated. In the present study, we examined the *n*-hexadecane biodegradation ability of twenty-one *Prototheca* sp., which were obtained from the Incorporated Administrative Agency National Institute of Technology and Evaluation Biological Resource Center (NBRC) and the Riken Bioresource Center Japan Collection of Microorganisms (JCM) in Japan, and identified derivatives derived from *n*-hexadecane. All *Prototheca* strains were cultivated in 5 ml of Medium GY (0.5% glucose and 0.5% yeast extract, pH 6.2) supplemented with 2% (v/v) *n*-alkane in a test tube at 28°C for 5 d with shaking (300 strokes/min), unless otherwise stated.

Total lipids were extracted from the reaction mixture with chloroform-methanol (1:2, v/v) according to the Bligh-Dyer method (9). The resultant lipids were analyzed with a GC-17A gas-liquid chromatograph (GLC; Shimadzu, Kyoto, Japan) equipped with a TC-WAX capillary column (0.25 mm I.D. \times 30 m; GL Sciences, Tokyo, Japan) and a flame ionization detector. The initial column temperature of 180°C was raised at 2°C/min to 240°C and then held for 10 min at 240°C. 1-Hexadecanol was used as an internal standard for quantitative analysis.

Total lipids were applied to a Sep-Pak Plus Silica Cartridge (Waters Co., Milford,

MA) to remove residual *n*-hexadecane. *n*-Hexadecane was eluted with 10 ml of *n*-hexane, and then other lipids including the derivatives derived from *n*-hexadecane were eluted with 10 ml of methanol. The collected fraction containing the derivatives was concentrated with a centrifugal evaporator. The lipids were separated by high performance liquid chromatography (HPLC, LC-10A system, Shimadzu) on a CLC-ODS (M) column (250 x 4.6 mm I.D.; Shimadzu). The mobile phase was acetonitrile-H₂O (8:2, v/v), the flow rate was 1.0 ml/min, and the effluent was monitored with a refractive index detector. The purified compounds were subjected to structural determination by means of gas chromatography-mass spectrometry (GC-MS) using a GCMS-OP5050 (Shimadzu) operating at an ionization voltage of 70 eV and proton nuclear magnetic resonance (¹H-NMR). ¹H-NMR and ¹H- two dimensional nuclear Overhauser effect spectroscopy (¹H-NOESY) analytical experiments were performed on a Bruker Biospin DMX-750 (750 MHz for ¹H; Bruker Co., Billerica, MA), and chemical shifts were assigned relative to the solvent signal. Compounds were dissolved in dichloromethane-d₂ and the diameter of the tube was 5 mm.

Total lipids of *Prototheca* sp. cultivated in Medium GY supplemented with 2% *n*-hexadecane were analyzed by GLC. Two unknown materials, designated as UK1 and UK2, were detected as major compounds on GLC chromatography for *P. zopfii* JCM9400 and *P. zopfii* NBRC6998, respectively (Fig. 1). These compounds were assumed to be derivatives of *n*-hexadecane due to their detection with just addition of *n*-hexadecane to the medium. UK1 and UK2 from *P. zopfii* JCM9400 and *P. zopfii*

Fig. 1

NBRC6998, respectively, were purified by means of HPLC for their structural identification. They were analyzed by means of GC-MS, as shown in Fig. 1. The molecular ion peak of UK1 was detected at m/z 240 (Fig. 1C). The fragment ion peak at m/z 183 was characteristic of 5-hexadecanone. The fragment ion peak pattern of UK1 corresponded to that of commercially available 5-hexadecanone used as an authentic standard. The fragment ion peak of UK2 at m/z 224 indicated its molecular ion peak, from which the molecular weight of H_2O is deducted, as shown in Fig. 1D. The fragment ion peak at m/z 185 was characteristic of 5-hexadecanol. The fragment ion peak pattern of UK2 corresponded to that of commercially available 5-hexadecanol as an authentic standard. UK1 and UK2 were purified and then analyzed by 1H -NMR (see supplementary data). The signals (2.38 ppm, *m*, 4H; 1.56 ppm, *m*, 4H) of UK1 indicated the existence of a ketone group. The signal (3.59 ppm, *m*, 1H) of UK2 indicated the existence of a hydroxyl group characteristic of a secondary alcohol. These results corresponded to those for each authentic standard on 1H -NMR analysis. UK1 and UK2 were identified as 5-hexadecanone and 5-hexadecanol, respectively.

We measured time courses of decrease of *n*-hexadecane and accumulation of 5-hexadecanol and 5-hexadecanone during the cultivation of *P. zopfii* JCM9400 in the medium containing 2% (v/v) *n*-hexadecane (Fig. 2). *n*-Hexadecane gradually decreased and its concentration led to 0.2% at 6th day. The concentrations of 5-hexadecanol and 5-hexadecanone reached 1.1 mg/L of broth at 3rd day and 82.6 mg/L of broth at 4th day, respectively, followed by decrease of their amounts with a parallel to

Fig. 2

the decrease of *n*-hexadecane. These oxidized products were suggested to be derived from *n*-hexadecane. On the other hand, we recognized the generation of pentadecanoic or hexadecanoic acid in addition with 1-pentadecanol or 1-hexadecanol to the culture medium, respectively (data not shown). This indicates that *P. zopfii* JCM9400 has a conversion activity of a primary fatty alcohol to the corresponding fatty acid. Even if *n*-pentadecane was added to the culture medium for *P. zopfii* JCM9400, we detected neither 1-pentadecanol nor pentadecanoic acid in the total lipids prepared according to the Bligh-Dyer method (data not shown). This indicates that *P. zopfii* JCM9400 has no conversion activity of pentadecane to 1-pentadecanol as the first step of terminal oxidization for *n*-alkanes.

Twenty-one strains of *Prototheca* sp. obtained from microbial type culture collections in Japan were examined as to their *n*-hexadecane degradation abilities. Some of them, capable of using *n*-hexadecane as a carbon source, accumulated 5-hexadecanone and 5-hexadecanol (Table 1). *Prototheca wickerhamii*, *Prototheca moriformis*, and *Prototheca stagnora* JCM9641 did not accumulate any derivatives derived from *n*-hexadecane. *Prototheca zopfii* JCM9400 exhibited the highest production (238.3 mg/L of cultural broth) of 5-hexadecanone. *P. zopfii* NBRC6998, *P. zopfii* NBRC7532, *P. zopfii* NBRC7533, and *Prototheca eriobotryae* NBRC32449 exhibited high 5-hexadecanol, rather than 5-hexadecanone, production.

Table 1

Thus far, microbial degradation of hydrocarbons through the subterminal oxidation pathway has been reported. Bacteria and many methanotrophs co-oxidize

short-chain alkanes via terminal as well as subterminal oxidation (10, 11). Subterminal oxidation has also been found for longer alkanes in *Penicillium*, *Bacillus*, *Pseudomonas* (12), and *Rhodococcus* sp. (13). However, microorganisms that accumulate 5th-positional-oxidation derivatives of *n*-hexadecane have not yet been reported. In the present study, we first found that *Prototheca* sp., especially *P. zopfii* JCM9400, exhibit high *n*-hexadecane degradation abilities, and accumulate unique oxidative products such as 5-hexadecanone and 5-hexadecanol. In *P. zopfii* JCM9400, *n*-hexadecane is presumed to be oxidized to 5-hexadecanol and then to 5-hexadecanone through general subterminal oxidation steps. Fatty alcohols like 5-hexadecanol can be a component of waxes by formation of an ester with some acids. Ketones like 5-hexadecanone can be converted to epoxy compounds by oxidation to be used as resin materials. As shown in Fig. 1, minor derivatives other than those identified in this study are present in some *Prototheca* sp. We need to elucidate the subterminal oxidation pathway for *n*-hexadecane in *Prototheca* sp. through identification of the minor derivatives.

Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research (grant 24658078) to E.S. from the Ministry of Education, Science, Sports, and Culture of Japan.

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Figure legends

FIG. 1. GLC chromatograms of lipids from *Prototheca zopfii* JCM9400 (A) and *P. zopfii* NBRC6998 (B). GC-MS spectra and deduced structural formulae of UK1 (C) and UK2 (D). These strains were cultivated in medium supplemented with *n*-hexadecane.

FIG. 2. Time courses of concentrations of *n*-hexadecane (solid circle), 5-hexadecanol (open triangle), and 5-hexadecanone (open square) on the cultivation of *P. zopfii* JCM9400 in the medium containing 2% (v/v) *n*-hexadecane.

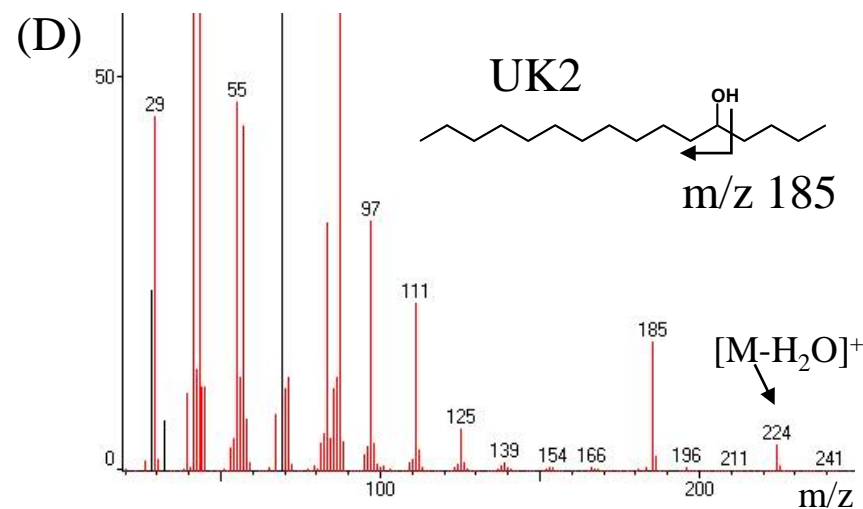
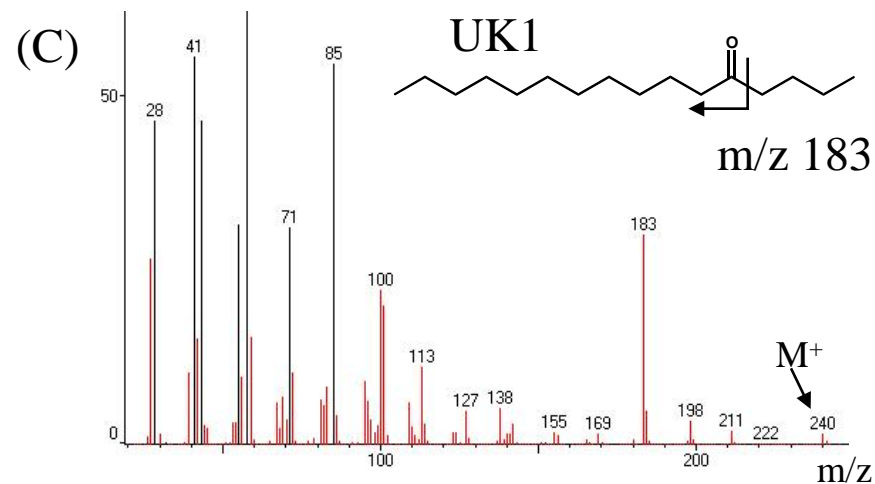
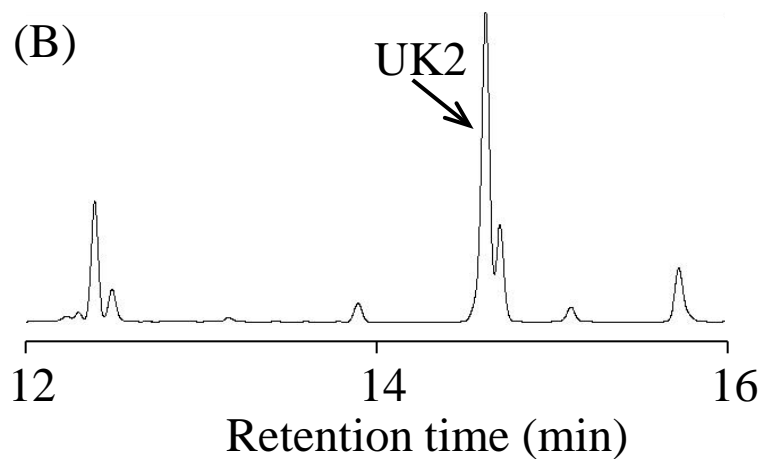
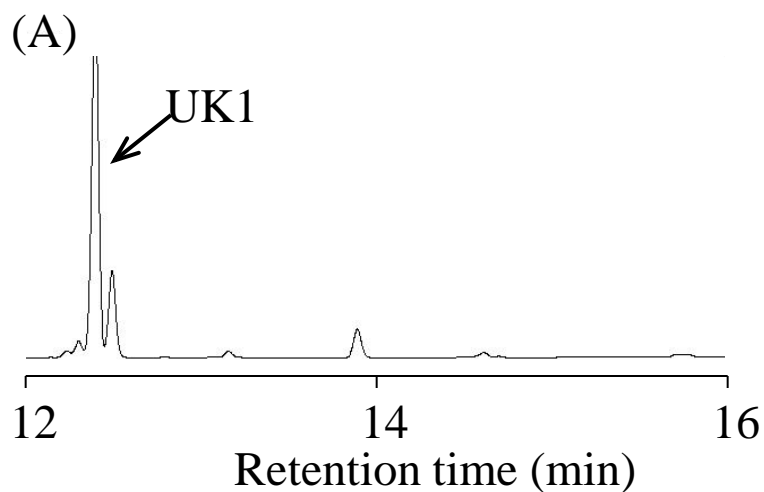


Fig. 1. Sakuradani et al.

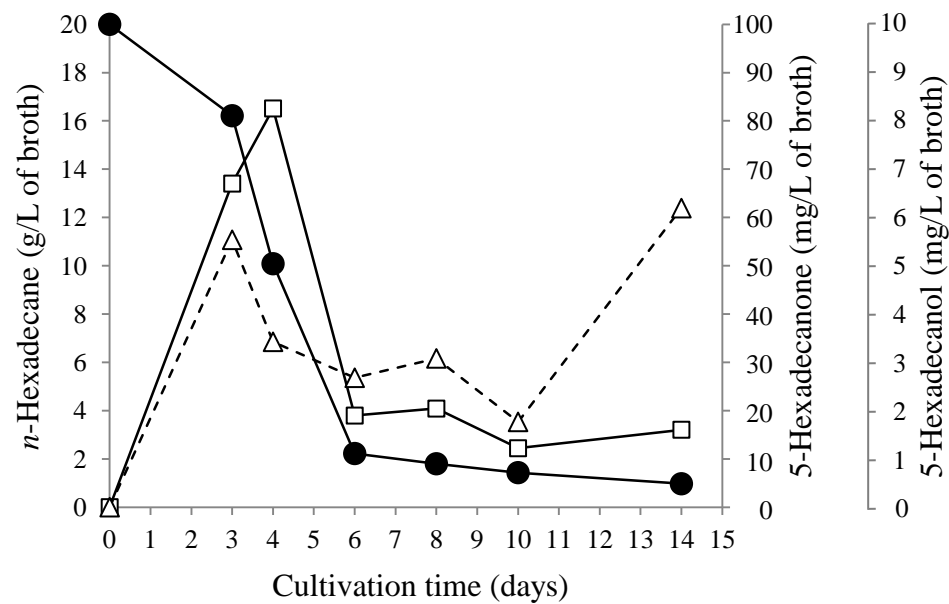


Fig. 2. Sakuradani et al.

TABLE 1. Comparison of the amounts of 5-hexadecanone and 5-hexadecanol produced by *Prototheca* sp.^a

Strain	5-Hexadecanone (mg/L)	5-Hexadecanol (mg/L)
<i>Prototheca zopfii</i> JCM9400	238.3	6.8
<i>P. zopfii</i> JCM9646	10.3	16.6
<i>P. zopfii</i> NBRC6998	14.2	22.6
<i>P. zopfii</i> NBRC7532	2.1	26.3
<i>P. zopfii</i> NBRC7533	2.5	22.2
<i>P. zopfii</i> NBRC7534	3.0	13.1
<i>P. zopfii</i> NBRC7535	43.3	7.8
<i>P. zopfii</i> NBRC7536	46.8	9.0
<i>P. trispora</i> NBRC6996	108.0	11.4
<i>P. eriobotryae</i> NBRC32449	13.8	26.1
<i>P. thermoturica</i> JCM8557	13.5	2.2
<i>P. stagnora</i> JCM9641	- ^b	-
<i>P. stagnora</i> JCM9642	11.0	2.6
<i>P. portoricensis</i> NIES-2182	2.3	5.9
<i>P. moriformis</i> JCM9640	-	-
<i>P. moriformis</i> JCM9729	-	-
<i>P. moriformis</i> NBRC6995	-	-
<i>P. wickerhamii</i> JCM9643	-	-
<i>P. wickerhamii</i> JCM9644	-	-
<i>P. wickerhamii</i> JCM9645	-	-
<i>P. wickerhamii</i> NBRC6997	-	-

^a*Prototheca* sp. were cultivated in Medium GY supplemented with *n*-hexadecane (final concentration, 2%).

^b-, not detected.

Supplemental data 1

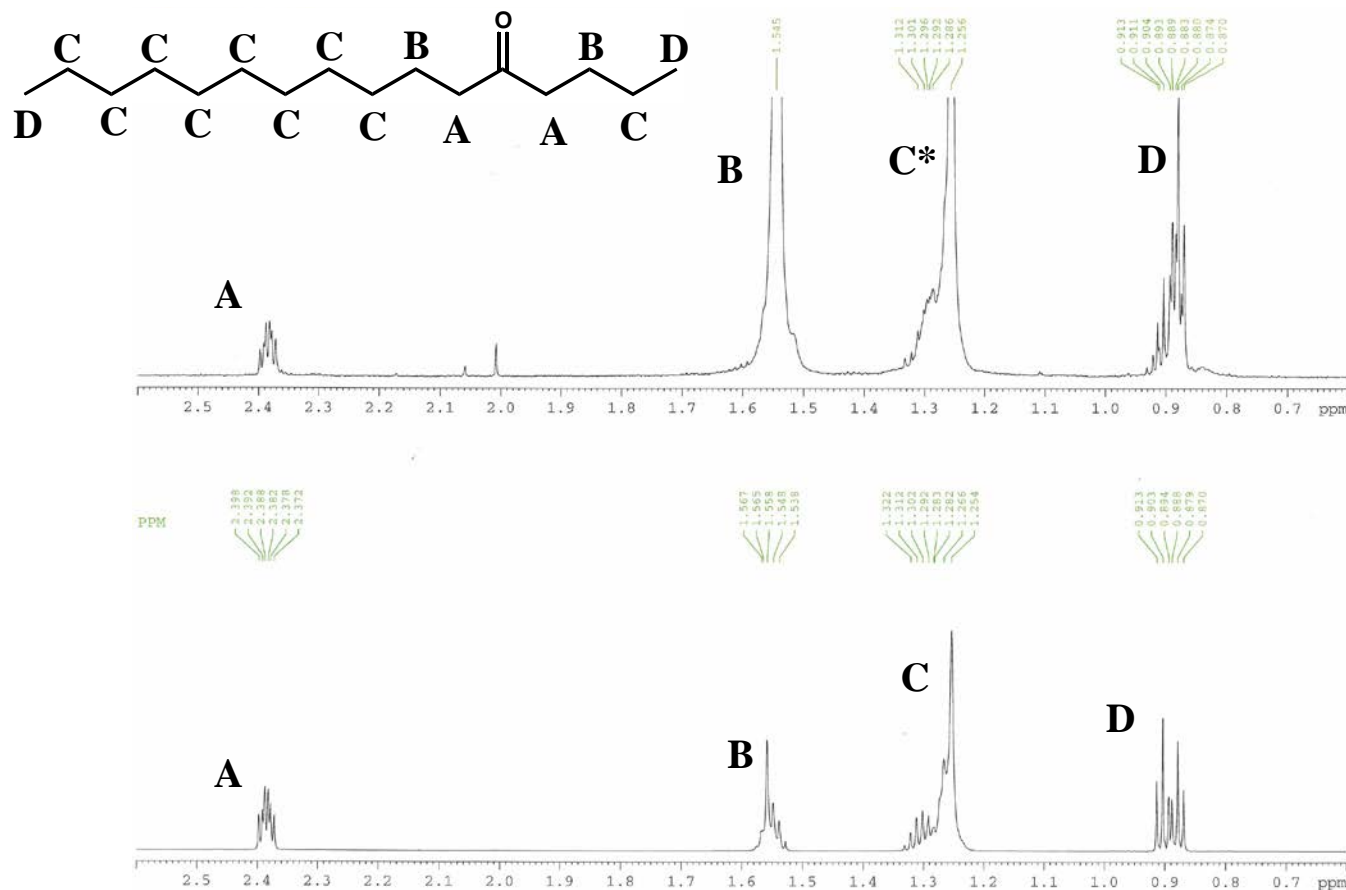


FIG. S1. Comparison of ¹H-NMR spectra of UK1 (top) and authentic 5-hexadecanone (bottom). The letters (A-D) indicate the protons corresponding to individual peaks. The C spectrum of UK1 (top) contains a signal derived from H₂O as an impurity.

Supplemental data 2

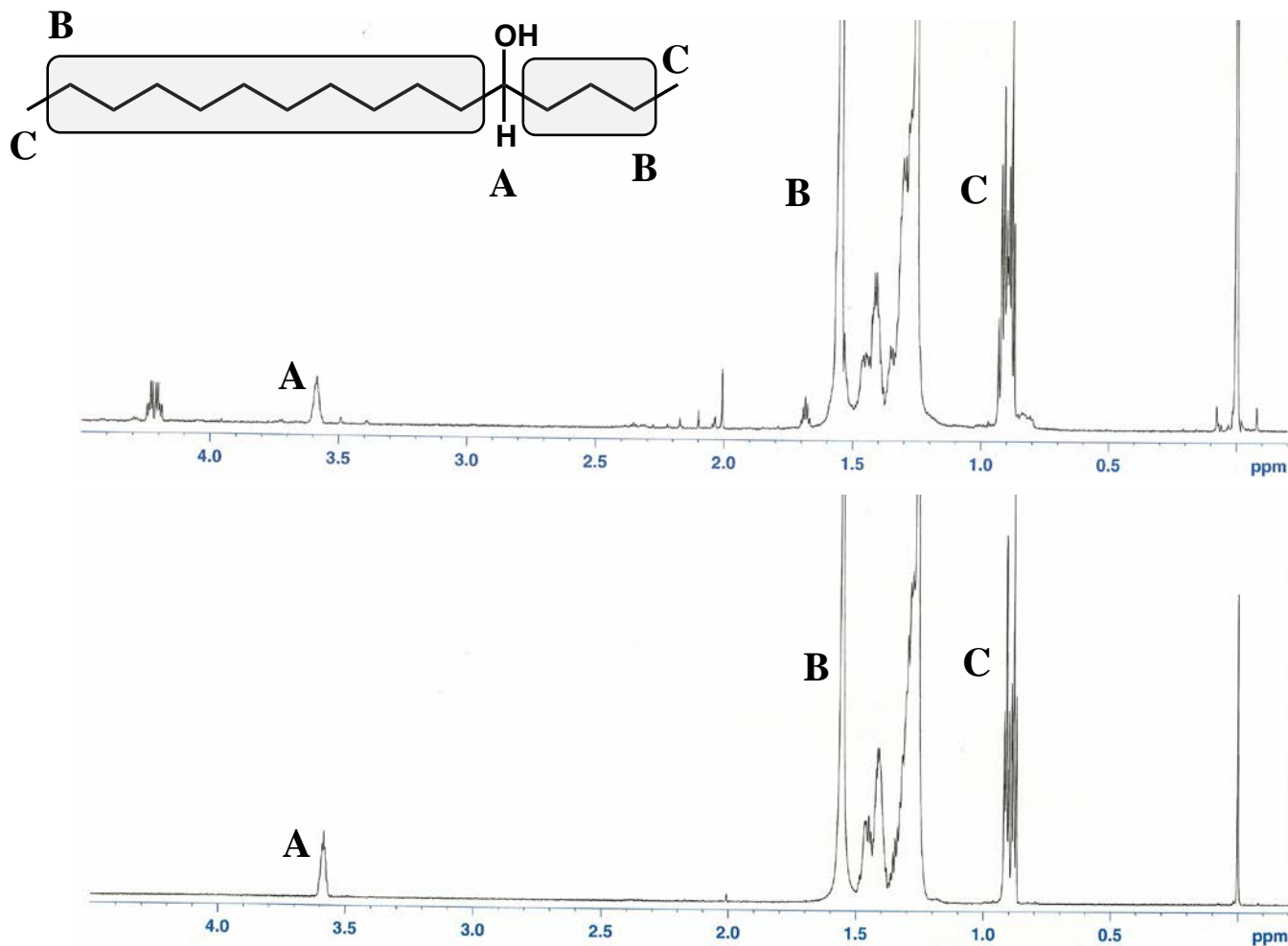


FIG. S2. Comparison of ^1H -NMR spectra of UK2 (top) and authentic 5-hexadecanone (bottom). The letters (A-C) indicate the protons corresponding to individual peaks.